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Energy efficient COD and N-removal from high-strength wastewater by a passively aerated GAO dominated biofilm

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Abstract

Conventional aerobic treatment of high-strength wastewater is not economical due to excessively high energy requirement for compressed air supply. The use of passive aeration avoids the use of compressed air and enables energy efficient oxygen supply directly from the air. This study evaluates a passively aerated simultaneous nitrification and denitrification performing biofilm to treat concentrated wastewater. The biofilm reactor was operated >5-months under alternating anaerobic/aerobic conditions. For 4-times concentrated wastewater, >80% COD (2307 $mgL^{-1}h^{-1}$) and >60% N (60 $mgL^{-1}h^{-1}$) was removed at a hydraulic retention time (HRT) of 7h. A double application in the same reactor enabled > 95% COD and 85% N-removal, at an overall HRT of 14h which is substantially shorter than what traditional activated sludge-based systems would require for the treatment of such concentrated feeds. Microbial community analysis showed *Candidatus competibacter* (27%) and nitrifying bacteria (*Nitrosomonas,* and *Nitrospira*) as key microbes involved in COD and N-removal, respectively.

Keywords: Zeolites, Bioregeneration, Simultaneous nitrification and denitrification, Poly-hydroxyalkanoate (PHA), Low-energy wastewater treatment.

1. Introduction

High-strength industrial wastewater (such as wastewater from animal farms and food processing facilities) is a major source of water pollution because of its elevated concentration of organic compounds and nutrients (Bustamante & Liao, 2017). Therefore, wastewater needs to be treated before its disposal into the environment. Conventional biological wastewater treatment technologies principally rely on aerobic processes in which microorganisms degrade the soluble and colloidal organic materials and reclaim the water (Tchobanoglous et al., 2014). However, despite its high organic carbon and nutrient removal efficiency, aerobic activated sludge treatment processes have major limitations such as the generation of excessive amounts of sludge and high energy requirement (McCarty et al., 2011; Tchobanoglous et al., 2014).

As an alternative to the aerobic wastewater treatment technologies, anaerobic digestion (AD) provides a more suitable option for the treatment of high-strength wastewater as it requires no oxygen and produces less excess sludge. Moreover, the AD has the potential to recover the used energy by generating biogas; hence, making the treatment process energy neutral or even energy positive (Aponte-Morales et al., 2016; McCarty et al., 2011). However, the low growth rate of anaerobic microorganisms, a low settling rate of biomass, high sensitivity to toxic shock loadings and fluctuation in environmental conditions often lead to failure of the AD treatment process (Bustamante & Liao, 2017). Another limitation is that N-removal cannot be achieved by the anaerobic digestion process, and this results in effluent quality that usually fails to comply with standards (Ahammad et al., 2013; Aponte-Morales et al., 2016).

A separate technology that enables significant oxygen savings for N-removal from high strength wastewater makes use of Anammox bacteria that can convert ammonium and nitrite to N_2 in the absence of oxygen (Kartal et al., 2013; Third et al., 2005b). This technology requires a low carbon level (or low C/N ratio) and is proposed for the side-stream treatment of NH_4^+-N rich wastewaters such as effluent of anaerobic digesters (Ali & Okabe, 2015). By combining this anaerobic step with partial nitritation and ORP control, up to 220 mg L^{-1} d^{-1} of N-removal was described to be possible (Zekker et al., 2018), However, the mainstream application of anammox technology is limited due to the extremely slow growth rate of bacteria resulting in longer start-up period and the high C/N ratio of municipal wastewater (Ali & Okabe, 2015; Zekker et al., 2012).

Biofilm-based wastewater treatment technology has shown great potential for the treatment of wastewater because of its operational flexibility, low sludge production and smaller plant size (Ma et al., 2017). A number of biofilm-based wastewater treatment technologies have been developed so far, such as the sequencing batch biofilm reactor (SBBR) where nitrogen removal can be achieved in a single reactor by a process called simultaneous nitrification and denitrification (SND) (Ma et al., 2017). The dynamic feast (anaerobic) and famine (aerobic) conditions of SBBR favors the development of storage microorganisms such as polyphosphate accumulating organism (PAO) and glycogen accumulating organism (GAO) (van Loosdrecht et al., 1997).

Polyphosphate accumulating organism uptake organic carbon anaerobically and convert it into PHA. The energy for this conversion comes from the hydrolysis of polyphosphate and glycogen. In a subsequent aerobic phase, PAOs can oxidize intracellularly stored PHA to generate energy for phosphorus uptake and its polymerization as polyphosphate (Mino et al., 1998; van Loosdrecht et al., 1997). Glycogen accumulating organisms can also uptake and store soluble organic carbon as PHA under anaerobic condition, and when O_2 or NO_3^--N becomes available use the stored PHA for glycogen replenishment and cellular growth (Liu et al., 1996; Zhou et al., 2008). While glycogen accumulating organisms (GAOs) do not contribute to the phosphorus removal and compete with PAOs for substrate (Liu et al., 1996; Oehmen et al., 2006; Zhou et al., 2008), they are considered as undesirable microorganisms in biological nutrient removal porcesses.

Recently, GAOs were found to play an important role in efficient removal of organic carbon from wastewater in a biofilm process operated under sequential anaerobic and aerobic conditions (Flavigny & Cord-Ruwisch, 2015; Hossain et al., 2018; Hossain et al., 2017b). By periodic draining of the reactor, the biofilm that has stored COD as PHA takes up oxygen directly from the air (passive aeration). A more recent study (Cheng et al., 2018; Cord-Ruwisch et al., 2018; Hossain et al., 2017a) has described how, by the inclusion of zeolite as ammonium adsorbent in the GAO biofilm, also nitrogen could be removed. After anaerobic adsorption of ammonium onto the zeolite, the subsequent air exposure enabled the biofilm to regenerate zeolite during passive aeration by involving simultaneous nitrification and denitrification reactions. The described laboratory process termed passive aeration simultaneous nitrification and denitrification (PASND) enabled removal of organic carbon and nitrogen from wastewater by avoiding the energy-intensive transfer of oxygen into the bulk wastewater; thus, reducing the aeration energy cost of a wastewater treatment plant. Since the energy expense for aeration is roughly proportional to the strength of the wastewater, the above described biofilm process capability to treat more concentrated wastewaters is highly desirable.

The current study aims to evaluate and optimize the capacity of the PASND biofilm process to treat concentrated wastewater for which traditional aerobic treatment methods would require excessively high energy input. Moreover, the stability of the process and the response of microbial communities to the increased organic and nitrogen concentrations were also assessed.

2. Materials and Methods

2.1. Zeolite

The natural zeolite used in this study was obtained from a local supplier (Zeolite Australia Pty. Ltd.). The zeolites were repeatedly washed with deionized water to remove adhering dirt and soluble impurities, dried at 105°C for 24 h. The dried zeolite was ground in a milling machine to a fine powder and passed through British Standard Sieves (BSS). Zeolite particles of 75 µm was used in the current study.

2.2. Experimental setup and reactor operation

A tubular laboratory-scale reactor made up of methyl methacrylate with a working volume of 0.755 L was used in this study (Figure 1). The reactor was filled (20 % $V_{carrier}/V_{reactor}$) with packing material (AMB^{TM} Biomedia Bioballs) containing biofilm enriched with glycogen accumulating organism (GAO) as described in Hossain et al. (2017b). Then, 10 g of the zeolite powder (75 µm) was suspended in synthetic wastewater solution and trickled over the GAO biofilm coated packing materials for 24 h until most of the suspended zeolite powder (> 99 %) was adsorbed onto the biofilm (indicated by the optical density). In order to introduce nitrifying bacteria into the biofilm that could be emulated in real plants, 100 mL of activated sludge (collected from Subiaco Wastewater Treatment Plant, Western Australia) was trickled over the biofilm for 24 h. This zeolite amended hybrid biofilm reactor which aims at enabling simultaneous nitrification and denitrification by using oxygen directly from the air (passive aeration) is hereafter referred to as passive aeration SND (PASND) reactor. Operating temperature of the reactor was maintained at 25 ± 2°C.

In the current study, the PASND biofilm reactor was operated continuously for a period of more than 5-months. After 120 days of operation with normal (1x) strength wastewater, the influent feed concentrations were stepwise increased to 2x and 4x (Table 1). The biofilm reactor was operated under alternating anaerobic/ aerobic conditions. During the submerged or anaerobic (feast) phase, the reactor was fully loaded with synthetic wastewater (Third et al., 2003) (full void volume) and remained in the anaerobic condition for a specific period to adsorb organic carbon and ammonium. The anaerobically treated wastewater was drained out of the reactor by gravity to begin the aerobic (famine) stage. The top of the reactor was open to enable oxygen passively entering the reactor, whereby the biofilm was exposed to atmospheric oxygen. To enable monitoring of nitrogenous compounds in the aerobic stage (after draining), a small volume of the residual liquid (about 20 mL) was slowly recirculated through the biofilm reactor from the top to the bottom. The aerobic and anaerobic phase lengths of the biofilm process varied with the strength of wastewater (Supplementary material).

2.3. Analytical parameters of interest

The concentrations of chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), and orthophosphate (PO_4^{3-}-P) in the influent and effluent of the biofilm reactor were regularly measured to monitor system performance. Ammonium, nitrite, nitrate, orthophosphate, and COD concentrations were determined in accordance with Standard Methods (APHA, 2012). The acetate was measured by Gas Chromatography (Agilent 7820A, USA) according to the method used in Hossain et al. (2017b). Freeze dried biomass was used to measure poly-hydroxyalkanoates (PHAs) and glycogen. PHAs were determined by the sum of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV), which were analyzed as previously reported (Smolders et al., 1994). Glycogen was analyzed according to the method used by Hossain et al. (2017b). Total suspended solids (TSS), and volatile suspended solids (VSS) were measured according to the Standard Methods (APHA, 2012). pH and DO were measured using a pH meter and a DO meter (Mettler-Toledo, USA), respectively.

2.4. Microbial community analysis

Biofilm samples were collected at a different time during the operational period to reveal the evolution of the microbial community of the biofilm. Genomic DNA was extracted from these samples using the Power Soil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instruction, and quantified by spectroscopic methods (NanoDrop 2000, Thermo Fisher Scientific, USA). Bacterial 16S rRNA genes were PCR-amplified with barcoded forward primer 515F and reverse primer 806R (Caporaso et al., 2012) For each sample, polymerase chain reaction was carried out in a 25 μL total volume including 2.5 μL of normalized total genomic DNA (5 ng/μL), 0.2 μM of each primer and 12.5 μL of 2x KAPA HiFi HotStart Ready Mix (Kappa Biosystems, USA). The PCR cycling protocol consisted of an initial denaturation step of 95°C for 3 min, followed by 35 cycles of DNA denaturation at 95°C for 30s, primer annealing at 55°C for 30s, strand elongation at 72°C for 30s, and a final elongation step at 72°C for 5 min. All samples were amplified in triplicate, pooled and visualized in agarose gel. Combined PCR products were purified using the AMPure XP beads (Beckman Coulter, USA) and final amplicon concentrations were quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). Amplicons from all sample were pooled in equimolar ratios and sequenced on the MiSeq platform (Illumina, USA).

The forward and reverse reads were merged and filtered based on minimum lengths and expected errors, as specified in the USEARCH pipeline (Edgar, 2010). To identify bacterial genera present in samples, operational taxonomic units (OTUs) were selected by clustering sequences at 97% similarity with the UPARSE algorithm and chimeric sequences were removed using UCHIME (Edgar, 2013). Taxonomy was assigned to OTUs against the Greengenes 16S database (August 2013 release) (DeSantis et al., 2006) in QIIME 1.9.1 (Caporaso et al., 2010) using the UCLUST algorithm (Edgar, 2010) with default parameters.

3. Results and Discussion

3.1. PASND reactor performance with increased strength wastewater

A zeolite amended glycogen accumulating organism dominated PASND biofilm system was established and operated under sequential anaerobic and aerobic schemes as described in Hossain et al. (Unpublished results). This biofilm reactor was shown to remove organic carbon (COD) and nitrogen (NH_4^+-N) efficiently from wastewater with low aeration energy expense. In order to evaluate the capacity of the described biofilm system to treat concentrated wastewater, a baseline trial with standard strength (hereafter referred to as single strength or 1x) synthetic wastewater was tested (Figure 2A). During the anaerobic period, the biofilm removed about 492 ± 6 and 29 ± 1.3 mg L^{-1} COD and NH_4^+-N, respectively. The GAOs were responsible for the COD uptake and its subsequent conversion to intracellular PHA, whereas NH_4^+-N removal was attributed to the presence of the added ion-exchange material (zeolite) in the biofilm.

After subsequent draining, the NH_4^+-N adsorbed onto the zeolite particles was removed due to the occurrence of simultaneous nitrification and denitrification (SND) as previously described (Hossain et al., Unpublished results). As observed over many months of operation, SND was evident from the oxidation of adsorbed NH_4^+-N to the zeolite particles without significant accumulation of NO_2^--N and NO_3^--N (aerobic phase, Figure 2A) and from previously recorded N_2 build-up (Cheng et al., 2018). The anaerobic adsorption of ammonium onto zeolite particle represents N-removal from the wastewater solution. The subsequent aerobic conversion of adsorbed ammonium to N_2 via SND represents N-removal from the reactor. To enable long-term repeated operation, the quantity of N-adsorbed from the liquid equals that of N-removed from the zeolite via SND.

During aerobic stage, the intracellularly stored PHA acted as the electron source for denitrification, which offsets the acidification caused by nitrification. The persistence of denitrification during air exposure of the biofilm was attributed to oxygen depletion in the deeper layers (anoxic zones) of the very actively respiring biofilm. Overall, this baseline run shows the consistent performance of the system after more than 3-months of continuous operation, showing about 92 ± 2.5 % and 70 ± 1.5 % of COD and NH_4^+-N removal, respectively.

Subsequently, the ability of the PASND biofilm system to treat double (2x) strength wastewater was evaluated (Figure 2B). Doubling of feed concentration resulted in the doubling of the COD and NH_4^+-N load, as the anaerobic period was kept the same (2 h). Interestingly, the COD removal efficiency stayed at around 88 %, while the NH_4^+-N removal efficiency dropped to 66 %. The fact that similar COD and NH_4^+-N removal efficiencies were obtained irrespective of the strengths of wastewater suggests that the PASND biofilm system studied has the capability of treating even stronger wastewater. When quadruple strength (4x) wastewater was used, the biofilm system was found to remove about 1745 ± 27 mg COD L^{-1} and 117 ± 2.3 mg NH_4^+-N L^{-1} within 2 h of anaerobic phase (Figure 2C).

The COD uptake rate of the described PASND biofilm system increased significantly when concentrated wastewater was used. When using 4x strength feed, the initial anaerobic COD uptake rate was > 4-times higher than for normal strength (1x) feed reaching very high values of about 2307 mg L^{-1} h^{-1}. This can be explained by the high abundances of GAO (see below) and the high biomass (26 ± 1.7 g dry weight L^{-1} of reactor) content (the biomass content (VSS) of the PASND system remained relatively stable during trials with different feed concentrations). Such COD removal rate is exceptionally high compared to conventional wastewater treatment processes including up-flow anaerobic sludge blanket (UASB) reactor (62 mg L^{-1} h^{-1}) (Tandukar et al., 2007), activated sludge process (29 mg L^{-1} h^{-1}) (Tandukar et al., 2007) and even to high rate moving bed biofilm process (150 mg L^{-1} h^{-1}) (Brosseau et al., 2016).

If the above mentioned COD removal was by aerobic respiration (assuming 50 % COD assimilation) it would be equivalent to an oxygen uptake rate of 1154 mg L^{-1} h^{-1}, which would require an unrealistic oxygen mass transfer coefficient (kL_a value) of about 165 h^{-1} (for maintaining a dissolved oxygen level of 1 mg L^{-1} and assuming saturation value of 8 mg L^{-1}). Clearly anaerobic COD uptake including PHA synthesis by GAO biofilms offers substantially faster COD removal than by conventional activated sludge based process. However, the COD still requires oxidation by oxygen.

The total treatment time for different strength wastewater should also take the aerobic bioregeneration phase into account. The required duration of the aerobic period can be determined from the depletion of the NH_4^+-N adsorbed onto zeolite and consumption of the stored intracellular organic carbon (i.e., PHA). Aeration phases longer than that would lead to the undesired exhaustion of PHA, potentially limiting denitrification capability, as described for activated sludge systems (Third et al., 2005a). The nitrogen profiles during the aerobic period demonstrated that NH_4^+-N was completely oxidized within 1 h and 2.5 h for single and double strength wastewater, respectively with a negligible amount of nitrite and nitrate accumulation (aerobic phase, Figure 2A and 2B). However, compared with 1x and 2x feed, the complete NH_4^+-N oxidation during the aerobic stage of the 4x feed required substantially longer time (5 h) and resulted in measurable nitrite accumulation.

The accumulation of nitrite (12.7 ± 1.7 mg L^{-1}) in the 4x feed trial can be explained by the observation that denitrifying glycogen accumulating organism (DGAO) present in the biofilm seemed to prefer reduction of nitrate over nitrite (anoxic nitrate and nitrite reduction rates were 20.9 ± 2.7 and 11.8 ± 1.5 mg L^{-1} h^{-1}, respectively). This preference of GAO dominated biomass to reduce nitrate rather than nitrite has been reported in previous studies (Bassin et al., 2012; Ribera-Guardia et al., 2016). Eventually, all of the accumulated nitrite was reduced (Figure 2C) demonstrating the complete denitrification capability of the PASND biofilm also for the 4-times concentrated wastewater.

Overall, the above results demonstrated that higher strength (up to 4x) wastewater could be efficiently treated in the zeolite amended PASND biofilm reactor but did not require a proportionally longer treatment time (Supplementary material). Since the aerobic time requirement for zeolite bioregeneration varies with feed strength, the aerobic phase length was adjusted (Supplementary material) for stronger feeds during long-term operation of the biofilm system to demonstrate its process stability.

3.2. Intracellular carbon transformation

With increasing feed concentration from 1x to 4x the biomass almost proportionally stored more COD, over the same time period (2 h anaerobic period) (Figure 3A) leading to substantially higher COD uptake rates. The specific carbon uptake rate of the biofilm increased from 0.1 Cmmol g^{-1} VSS h^{-1} (1x wastewater) to 0.38 Cmmol g^{-1} VSS h^{-1} (4x wastewater). This result is in agreement with the finding of Chiou and Yang (2008) who reported that anaerobic COD storage by adapted biofilms increased more than 4-times when the influent COD concentrations were increased from 100 to 500 mg L^{-1}. The organic substrate removal rate in a biofilm system is principally controlled by diffusion (Fan et al., 2017). The fact that the COD uptake rate was about proportional to the COD concentrations is in line with Fick's law of diffusion and suggests that the biomass was not yet substrate saturated. Further it was also reported that for COD storing biofilms the diffusion coefficient increases with the influent organic concentration (Fan et al., 2017).

The COD uptake and storage as PHA over 2 h, for normal (1x) strength wastewater lead to about 92 % COD uptake. As expected, the percentage of COD removal was less for the stronger wastewater (Supplementary material). However, the total amount of COD taken up increased almost proportionally with the concentration of the feed, storing up to 6.5 Cmmol L^{-1} for 4x wastewater (Figure 3A). The enhanced organic carbon uptake by the biofilm resulted in an increase in the accumulation of intracellular storage material (i.e., PHA) from 2.7 to 9.3 Cmmol L^{-1} for 1x and 4x wastewater, respectively. This observation is in line with the findings of Chiou and Yang (2008) who found that increased influent carbon concentrations resulted in greater levels of

PHA accumulation in a biofilm that underwent sequential anaerobic and aerobic conditions.

The uptake of organic substrate into biofilms depends not only on the internal and external mass transfer but also on its biological transformation (Fan et al., 2017). For the single-strength wastewater, the GAO dominated biofilm stored PHA at a rate of about 0.06 Cmmol g^{-1} VSS h^{-1} which increased more than 3-fold to 0.21 Cmmol g^{-1} VSS h^{-1} when 4x wastewater was used. The faster PHA synthesis rate with higher COD loading drives the diffusion of organics into the GAO biofilm resulting in enhanced storage of PHA within a short period of 2 h. The greater availability of reducing material (i.e., PHA) (Figure 3B) was responsible for efficient bioregeneration of zeolite (via SND) during the aerobic condition when high-strength (2x and 4x) feed was used (Figure 2B and 2C) in the current experiment.

The anaerobic uptake of organic substrate requires energy (ATP) which is supplied either by the cleavage of intracellular Poly-P or glycogen fermentation in PAO or GAO dominated systems, respectively. The question arises where the additional energy comes from to store the higher amount of carbon in case of concentrated (2x and 4x) feed. As shown in Figure 3C, at the higher feed concentrations the biomass degraded significantly more glycogen, necessary for additional carbon uptake and storage as PHA. The Carbon of glycogen hydrolyzed per C of COD taken up (Gly/C ratio) of the biofilm stayed relatively constant at around 1.1, which is in line with other studies using GAO bacteria but 2-fold higher than that of PAO (Table 2). This observation confirms that glycogen solely acted as the energy source for storage of COD as PHA in the describe biofilm system.

3.3. Long-term performance of the biofilm system

In order to assess the stability of the process, the performance of the zeolite amended PASND biofilm system was evaluated over long periods of continued automated operation. After 120 days of operation with normal strength (of which the last 20 days are shown here), 2x and 4x concentrations were used for 20 days each (Table 1, Figure 4). As the cycle length for the 2x and 4x feed was 5.5 and 7 h, respectively (Supplementary material), the system had run for 87 and 68 cycles, respectively. The duration of the anaerobic period was maintained the same (2 h) to investigate the performance of the system without proportionally increasing the reactor size. However, the length of the aerobic period varied based on previous results (Supplementary material) to enable complete bioregeneration of zeolite.

When concentrated feed was used in the PASND biofilm reactor, the COD removal efficiency decreased from 93 ± 1.4 % to about 88 ± 1.9 % and 81 ± 1.5 % for the 2x and 4x wastewater, respectively (Figure 4A). In contrast, the average organic removal rate (ORR) increased more than 200 % to 2920 ± 54 g COD m^{-3} d^{-1} (4x). This extremely high COD storage rate shows that the storage capacity of the biofilm was not exhausted at 1x feed. Further it can be explained by the increased abundance of GAO in the biofilm (Figure 5).

In terms of nitrogen removal, the PASND biofilm process showed a similar trend, where the NH_4^+-N removal efficiency slightly decreased to 62 ± 1.6 % for quadruple strength wastewater. Nevertheless, the nitrogen removal rate (NRR) of the biofilm system increased more than 2-times from 80 ± 1.4 g m^{-3} d^{-1} at 1x feed to 173 ± 5.2 g m^{-3} d^{-1} at 4x feed. This shows that for the amount of zeolite present in the biofilm system, the adsorption capacity was not exhausted, and that the zeolite concentration used was suitable for the treatment of high-strength wastewater.

One known effect of concentrated feeds is the potential inhibitory effect of ammonium on the activity of microorganisms (Aponte-Morales et al., 2016). The apparent lack of ammonium inhibition of the current study may be due to the presence of zeolite which maintains a low concentration of dissolved ammonium. He et al. (2007) reported that addition of zeolite powder minimizes the effect of ammonium shock loads to biomass. Nitrifying bacteria have been shown to readily concentrate on the surface of zeolite particles (due to the ease of accessible substrate) which accelerates the nitrification process (He et al., 2007). Further, the increased development of nitrifying organisms at the higher feed strengths (Figure 5) provides an additional explanation for adequate bioregeneration of zeolite during the aerobic phase and increased ammonium oxidation rates (Figure 2B and 2C). The bioregeneration efficiency of zeolite by nitrifier is well studied. In this study, it can be quantified indirectly by the repeated ammonium removal efficiency in the next cycle under anaerobic conditions.

3.4. Dynamic changes of microbial communities with increased wastewater concentrations

The biofilm samples (day 120, 140 and 159) were analyzed with high-throughput methods to reveal the shifts in overall microbial community composition with the increase in influent COD and NH_4^+-N concentrations. The initial bioinformatic analysis yielded a total of 219989 sequences which were assigned to different taxonomic levels. Overall, the richness of the bacterial community in the biofilm reactor declined after increasing the feed concentration, as evidenced by the decrease of both Chao 1 index and numbers of observed OTUs (Table 3). In addition, the microbial diversity also slightly decreased which is marked by the drop in Shannon index from 4.22 (day 120) to 4.02 (day 159). These results show that the increase in influent organic carbon and nitrogen concentrations diminished the diversity of the microbial community in the PASND biofilm system possibly by implying more extreme conditions, selecting more specifically adapted organisms.

The relative abundances of different phyla in the biofilm samples are shown in Supplementary material. *Proteobacteria* was the most abundant phylum in all the biofilm samples which accounted for > 35 % of the bacterial 16S rRNA gene sequences. Members of this bacterial group are considered important for wastewater treatment processes because of their role in carbon, phosphorous and nitrogen removal (Yang et al., 2014).

The relative abundances of the top 11 genera in the biofilm samples from the different operational periods are shown in Figure 5. The genus of *Candidatus competibacter*, and *Nitrospira* were distinguished by their increased relative abundances over the experimental period. The most notable change in the community composition was the increase in fraction of *Candidatus competibacter*, a known denitrifying glycogen accumulating organism (GAO). It increased from 20 % (day 120) to 27 % (day 159) as the influent wastewater concentrations increased 4-times within 40-days of continuous operation. The enhanced COD removal rate at increased organic loading rates, is attributed to the increase of relative abundances of GAO in the biofilm system and is evident by the positive correlation between *Ca*. *competibacter* and organic carbon removal rate. Members of the bacteria *Candidatus competibacter* have been reported to uptake organic carbon anaerobically and store as PHA, but the cells accumulate glycogen instead of poly-phosphate under aerobic conditions (Coats et al., 2011; McIlroy et al., 2014; Zeng et al., 2002).

Glycogen accumulating organisms are responsible for the denitrification reaction during the aerobic period since these bacteria are known to use their intracellular storage polymers (i.e., PHA, glycogen) as an electron source to reduce nitrite and/or nitrate (Bassin et al., 2012; Coats et al., 2011; Zhu et al., 2013). Most of the GAOs identified so far can use nitrate as an electron acceptor in addition to oxygen, but only members belonging to Clade I of Competibacter can use nitrite as well (Nielsen et al., 2010). The subgroups of *Candidatus competibacter* found in the biofilm samples were further analyzed using the clustering method of phylogenetic tree (McIlroy et al., 2014) and found that they are closely related to Competibacter Clade I (Supplementary material). The sequencing evidence of the presence of nitrirte reducing GAO is in line with the observation of nitrite reduction observed for the 4x feed trial (aerobic phase, Figure 2C).

Other microbial genera which showed a significant increase in their relative abundance includes *Nitrospira* (Figure 5). The fraction of the microbial community associated with *Nitrospira* increased by about 40 % when the influent NH_4^+-N concentrations increased 4-times over the experimental period. This observation is in accordance with the previous study (Tian et al., 2017) that observed that increases in ammonium feed concentration stimulated *Nitrospira* growth. While *Nitrospira* was traditionally seen as a nitrite-oxidizing bacterium (NOB), recent evidence established it as a complete nitrifier catalyzing ammonium oxidation to nitrate formation (Daims et al., 2015). It is interesting to note that the increased ammonium concentration in the more concentrated (2x and 4x) feed seems to have promoted *Nitrospira* development but did not affect the classical ammonium oxidizer *Nitrosomonas* (Figure 5). Whether this means that the PASND biofilm's bioregeneration of zeolite by ammonium oxidation is principally ascribed to *Nitrospira*, warrants a further investigation.

3.5. Practical implications

The present study demonstrated that the zeolite amended GAO biofilm system could be an effective treatment option for high-strength wastewater. However, using a single treatment, the proposed technology was unable to remove nitrogen from wastewater adequately (60 - 70% for 1x - 4x wastewater, Supplementary material) due to the limited capacity of the zeolite adsorbent and therefore requires further treatment. Such a subsequent treatment should not be done by a separate reactor as the lack of stored COD would limit denitrification and by that the occurrence of SND and zeolite bioregeneration. To solve this problem, the effluent was returned to the same reactor, after the completion of the aerobic phase, with the intent to make use of residual stored PHA available as the electron donor for denitrification.

When ammonium containing solution at equilibrium with zeolite was drained and re-entered into the same reactor after the normal aerobic phase length (between 1 and 5 h, Supplementary material), renewed ammonium adsorption was observed (Figure 6). However, as expected from Langmuir isotherms, the percentage ammonium adsorption of the repeat treatment was similar which is due to the efficient bioregeneration of zeolite as explained previously. Nevertheless, with two successive treatments using the same reactor, the total nitrogen removal efficiency for both double and quadruple strength wastewater could be increased to about 85 % (Figure 6B and 6C). This observation suggests that realistic N-removal by the zeolite amended, passively aerated biofilm process can be achieved also for high-strength wastewater.

The PASND process described here relies on passive aeration which itself avoids the energy costs associated with air blower usage of traditional aeration of submersed biomass. The amount of energy required to fill and drain the reactor has been calculated to be approximately 7.5 % of the energy necessary for compressed air supply by blowers for normal strength wastewater (Hossain et al., Unpublished results). For a double treatment this would save around 85 % of the energy costs of traditional activated sludge plants. Given that the energy requirement for compressed air supply in conventional wastewater treatment plants is approximately proportional to the concentration of the wastewater (Tchobanoglous et al., 2014), it can be deduced that the energy savings that can be made by using PASND increase substantially with the strength of the wastewater: the higher oxygen demand does not impact on the energy use of passive aeration. Overall, the results demonstrate that the described PASND biofilm reactor removes organic carbon and nitrogen from high strength wastewater without using energy intensive air compressors.

The PASND biofilm system was operated continuously for more than 5-months to investigate the stability of the process. During the experimental period, no loss of ammonium ion-exchange efficiency of the process was observed, and no fresh zeolite was added to the biofilm reactor. This result suggests that the bioregeneration efficiency did not decrease over time and zeolite was not lost from the biofilm. However, in real world application where inert suspended material can build up in the zeolite containing biofilm, some zeolite loss is expected and may need replacement. The amount of zeolite replacement needed will depend on the loss of suspended solids and biomass from the system and on the nature in which zeolite is retained in the solid phase of the reactor. In order to assess the frequency of zeolite addition into the PASND biofilm system, a further study is warranted.

For the purpose of reproducibility, this study has used artificial wastewater that has been used in the majority of similar studies. The efficiency of the process when operating with real wastewater and at a large scale needs to be established prior to accepting the efficiency gains found in this study. In particular, the presence of suspended solids, organic nitrogen and of complex organic substrates on the COD and N-removal capacity during the anaerobic phase should be investigated.

4. Conclusions

- The treatment of high-strength wastewater in the zeolite amended PASND biofilm reactor does not require a proportionally longer treatment time. The anaerobic phase can be similar for different strength feed, while the aerobic bioregeneration times should be adjusted according to feed strength.
- The application of PASND process could offer extensive cost-savings for aeration energy, in particular for concentrated wastewater streams for which an effective and economic treatment option does not exist.
- The PASND process offers a suitable rapid pre-treatment of concentrated wastewater to remove the majority of COD, ammonium with low-cost passive aeration for a subsequent traditional activated sludge treatment.

Acknowledgements

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Appendix A: Supplementary Material

E-supplementary data for this work can be found in e-version of this paper online.

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Table 1: Operational conditions of the biofilm reactor system

Wastewater concentration	Synthetic wastewater*	
	COD (mg L^{-1})	NH_4^+ - N (mg L^{-1})
1x (day 0 - 120)	532.4 ± 25.5	42.4 ± 1.7
2x (day 121 - 140)	1079.8 ± 47.3	85.7 ± 2.3
4x (day 141 - 160)	2141.0 ± 29.6	169.0 ± 3.3

* Other constituents of synthetic wastewater were kept unchanged throughout the experimental time (Supplementary material)

Table 2: Comparison of the anaerobic stoichiometric parameters observed in the present study with that of other reports

References	P/C (Pmmol/ Cmmol)	PHA/C (Cmmol/ Cmmol)	PHB/C (Cmmol/ Cmmol)	PHV/C (Cmmol/ Cmmol)	PHV/ PHB	Gly/C (Cmmol/ Cmmol)
This Study						
1x Feed	0.00	1.65	0.98	0.67	0.68	1.14
2x Feed	0.00	1.51	0.88	0.63	0.72	1.12
4x Feed	0.00	1.43	0.75	0.68	0.91	1.09
GAO studies						
Lopez-Vazquez et al. (2007)	0.01	1.97	1.28	0.69	0.54	1.20
Zeng et al. (2002)	NA	1.85	1.36	0.46	0.34	1.12
Filipe et al. (2001)	NA	1.65	1.26	0.38	0.31	0.83
PAO studies						
Smolders et al. (1994)	0.50	1.33	1.21	0.12	0.10	0.50
Zhou et al. (2008)	0.62	1.25	1.18	0.07	0.06	0.46
Acevedo et al. (2012)	0.70	1.36	1.31	0.05	0.04	0.38

Table 3: Community diversity indexes in different biofilm samples

Samples	No of sequences	Chao 1	Observed OTUs	Shannon index
Day 120	82231	1213.14	693	4.22
Day 140	48041	1175.94	688	4.05
Day 159	89717	1004.16	637	4.02

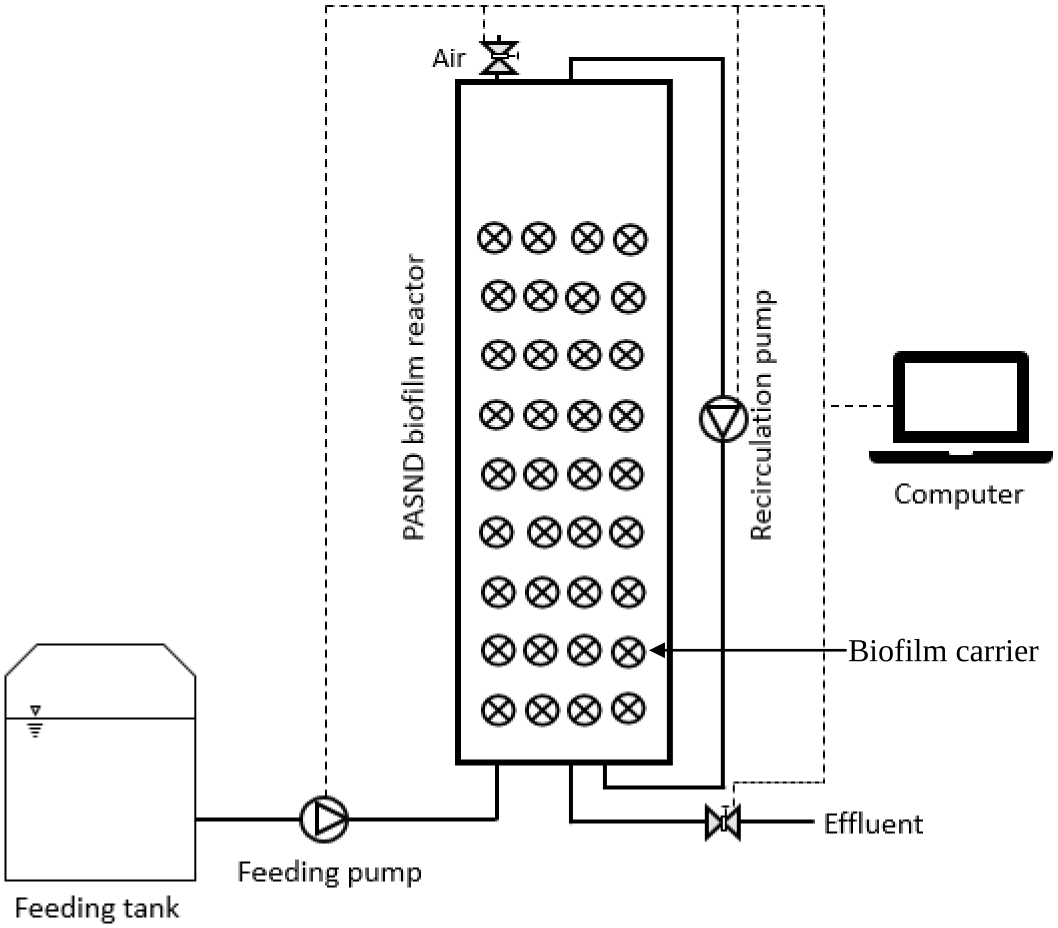
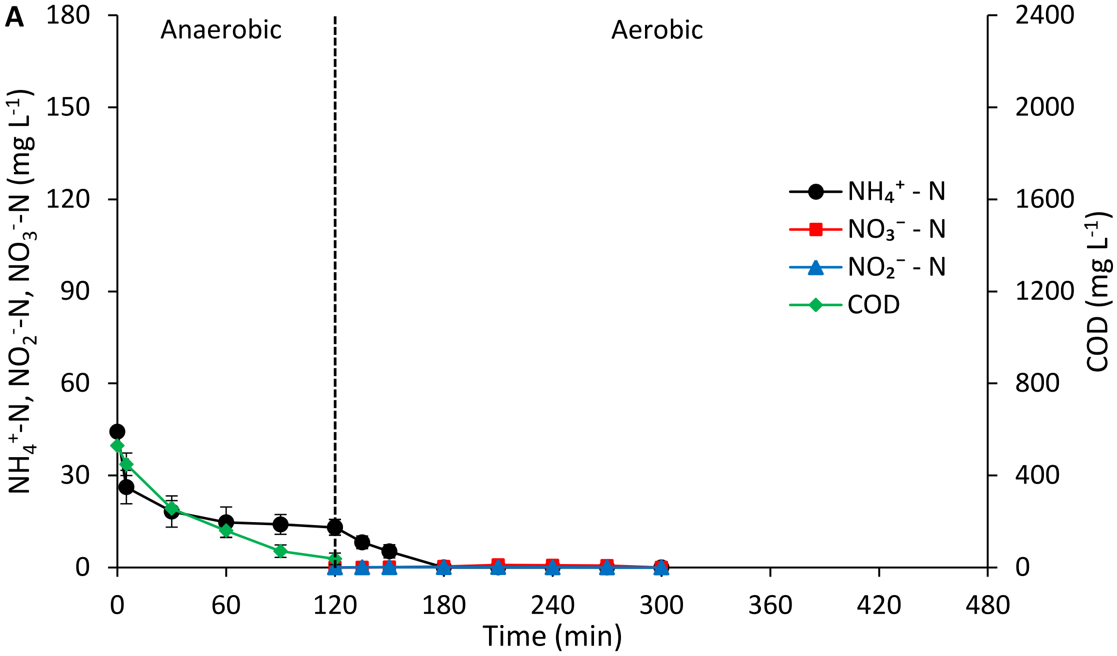
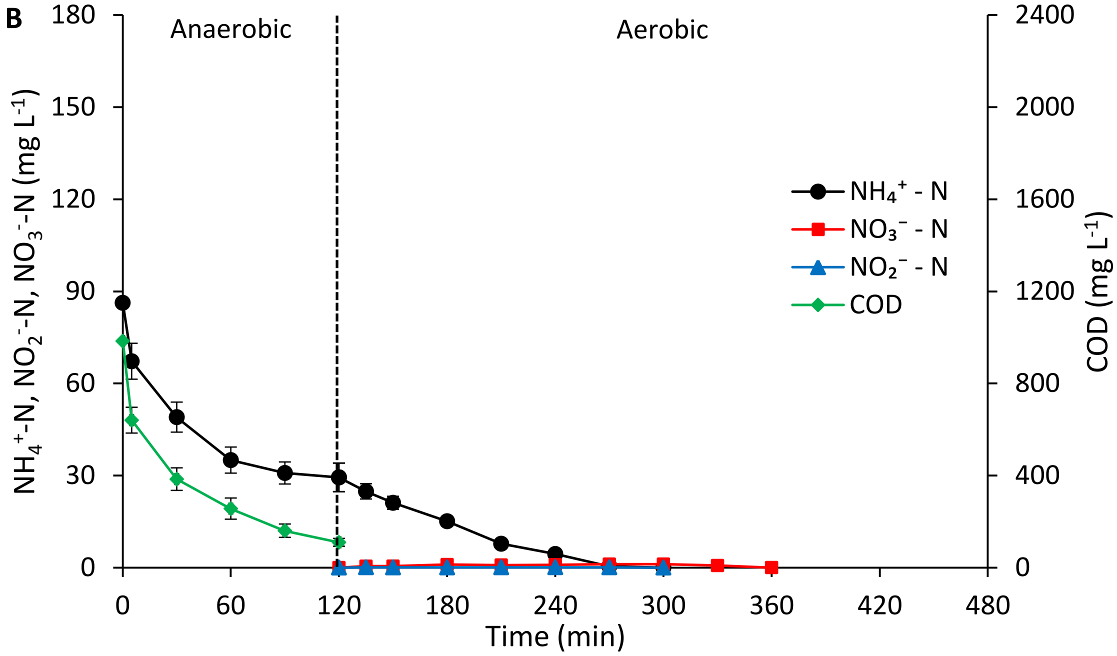


Figure 1: The schematic of the passive aeration simultaneous nitrification and denitrification (PASND) biofilm reactor. The biofilm grown on carrier materials was alternately exposed to wastewater (after flooding) and air (after draining) to facilitate anaerobic adsorption of COD (by GAO) and ammonium (to zeolite) and the subsequent aerobic bioregeneration of adsorption capacity.


A
Anaerobic
Aerobic
NH4+ - N
NO3− - N
NO2− - N
COD
NH4+-N, NO2−-N, NO3−-N (mg L-1)
COD (mg L-1)
Time (min)
180
150
120
90
60
30
0
2400
2000
1600
1200
800
400
0
0
60
120
180
240
300
360
420
480


B
Anaerobic
Aerobic
NH4+ - N
NO3− - N
NO2− - N
COD
NH4+-N, NO2−-N, NO3−-N (mg L-1)
COD (mg L-1)
Time (min)
180
150
120
90
60
30
0
2400
2000
1600
1200
800
400
0
0
60
120
180
240
300
360
420
480

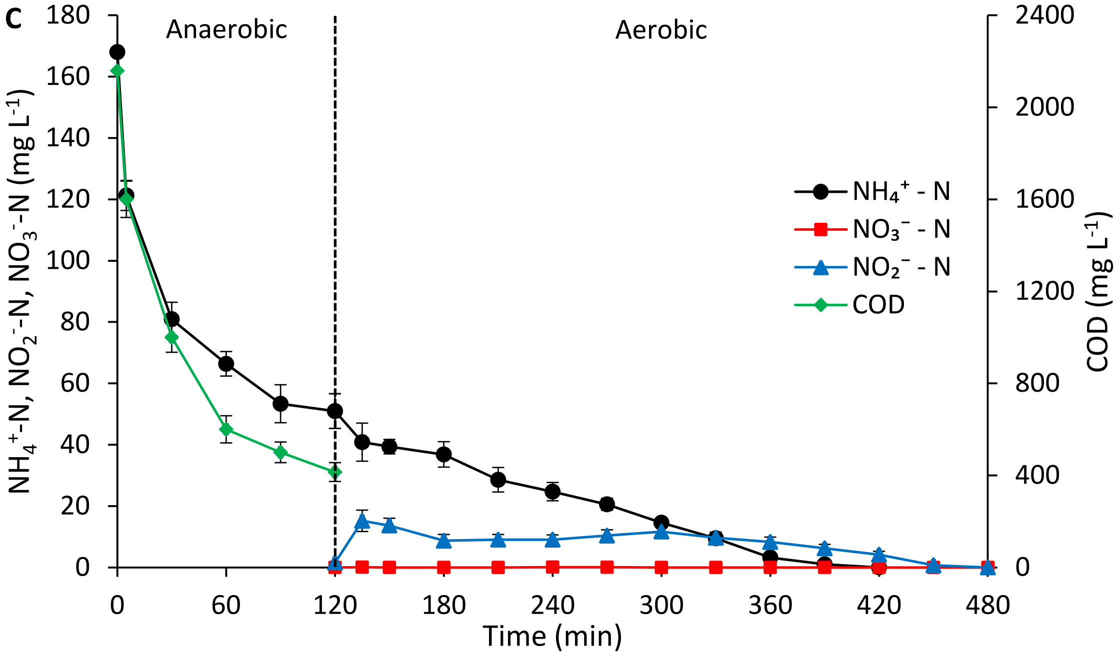


Figure 2: Observed conversions in the PASND biofilm system with (A) standard-strength (1x), (B) double strength (2x), and (C) quadruple strength (4x) wastewater. Measurements during the anaerobic phase are for concentrations in the bulk solution, and during the aerobic phase for the residual (around 10 % of drained solution) liquid retained to enable monitoring of biofilm activity.

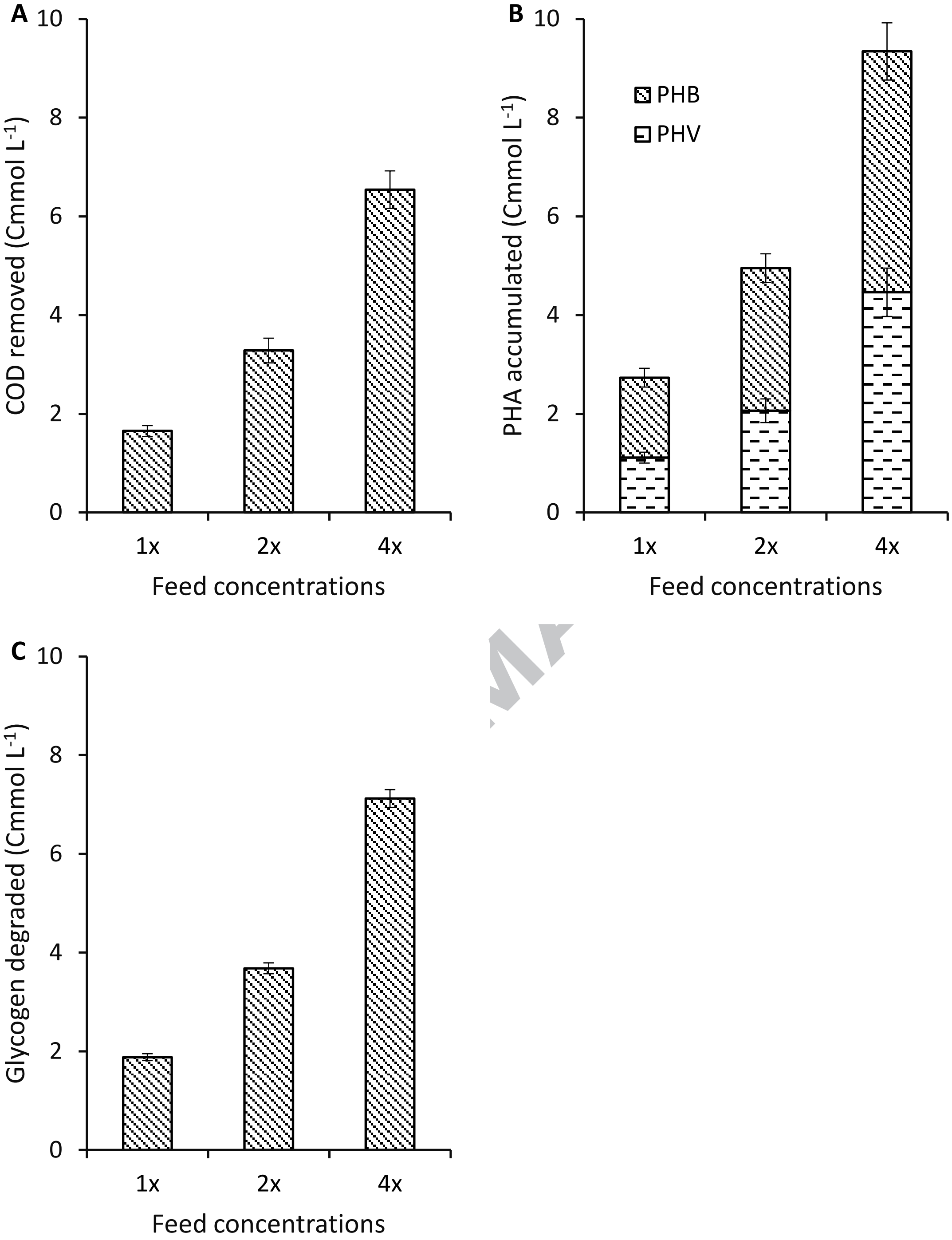


Figure 3: Carbon mass balance relationships between COD and the loss of glycogen and gain of PHA (PHB and PHV) during the anaerobic phase at different influent feed concentrations.

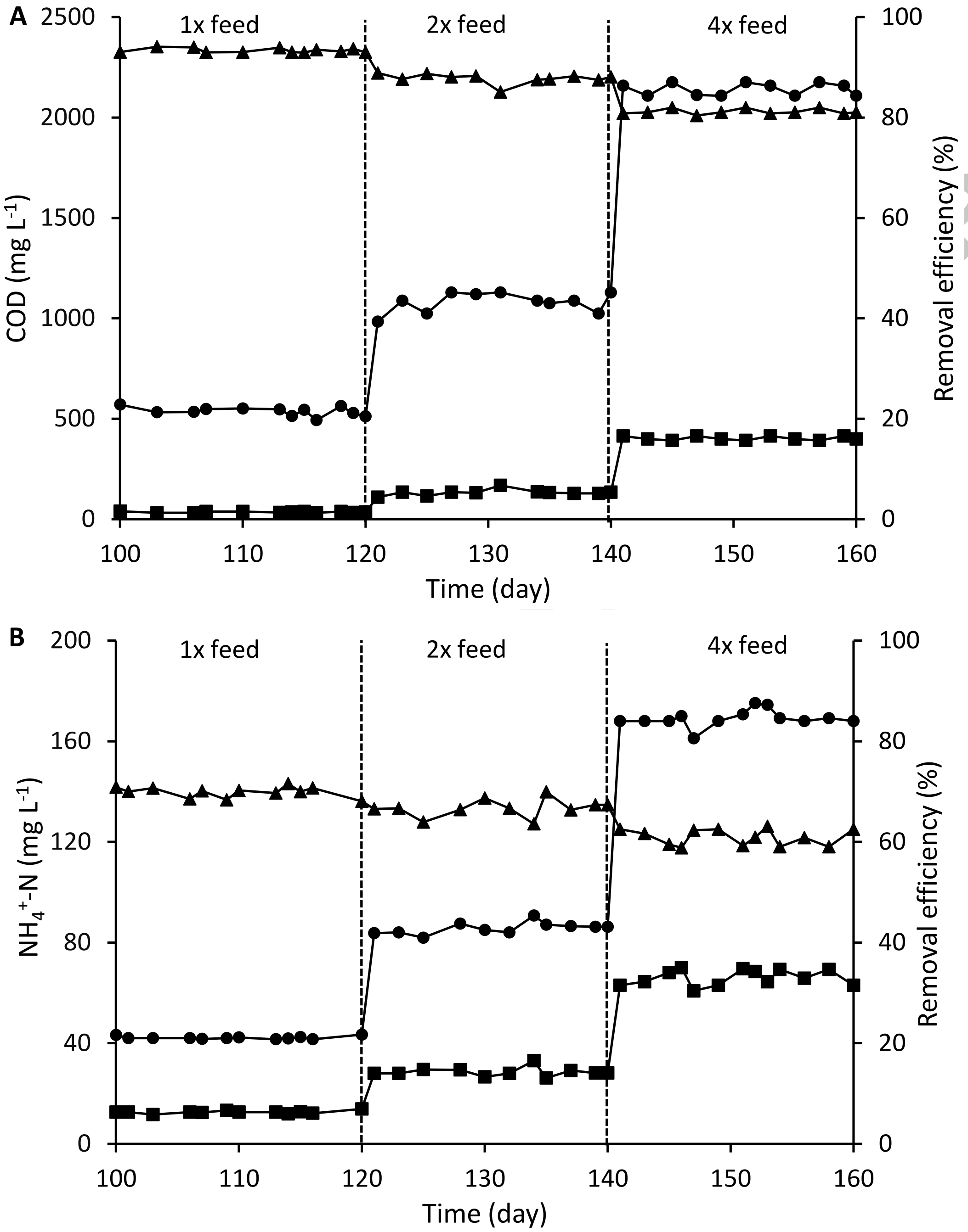


Figure 4: Removal of (A) organic carbon (COD) and (B) nitrogen in the biofilm system under different feed concentrations: (●) influent, (■) effluent and (▲) removal efficiency.

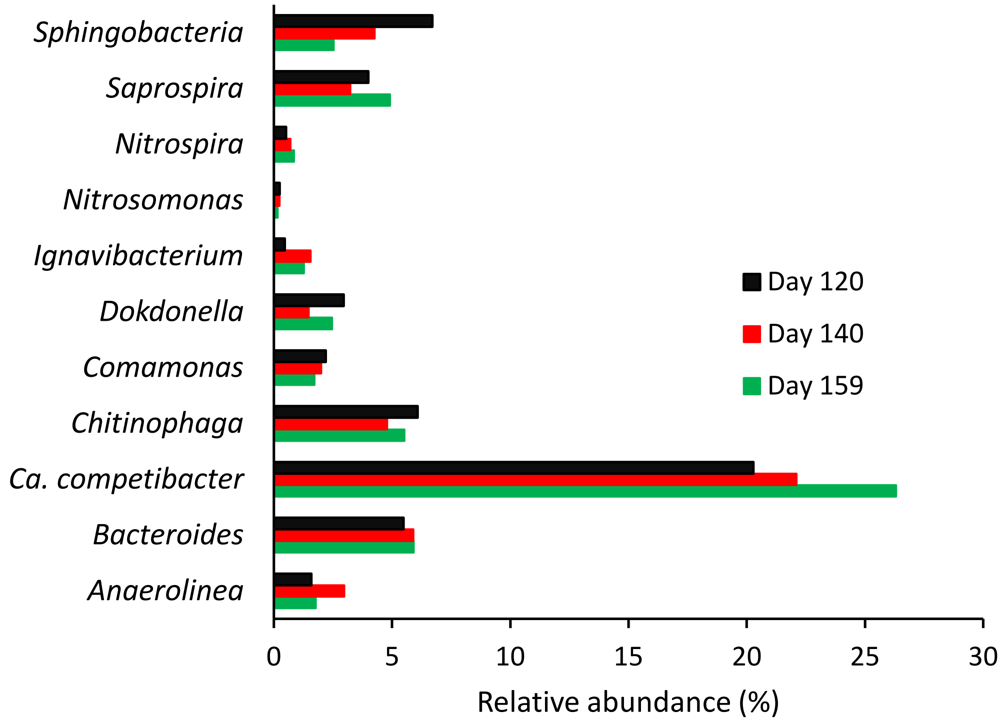
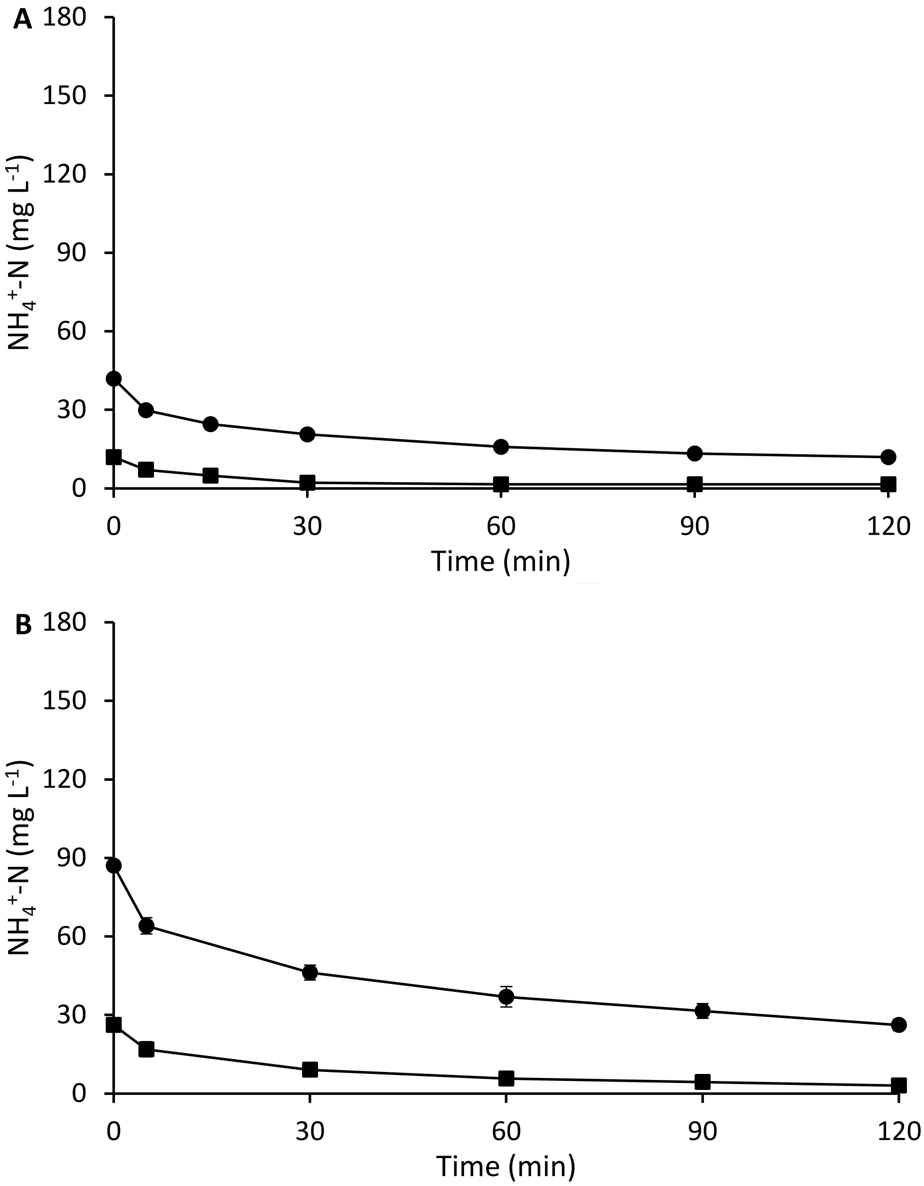


Figure 5: Relative abundances of the top 11 genera in the biofilm samples. The relative abundance for each genus was defined as the sum of OTUs assigned to a genus divided by the total OTUs of a biofilm sample.


A
180
150
120
90
60
30
0
NH4+-N (mg L-1)
0
30
60
90
120
Time (min)
B
180
150
120
90
60
30
0
NH4+-N (mg L-1)
0
30
60
90
120
Time (min)

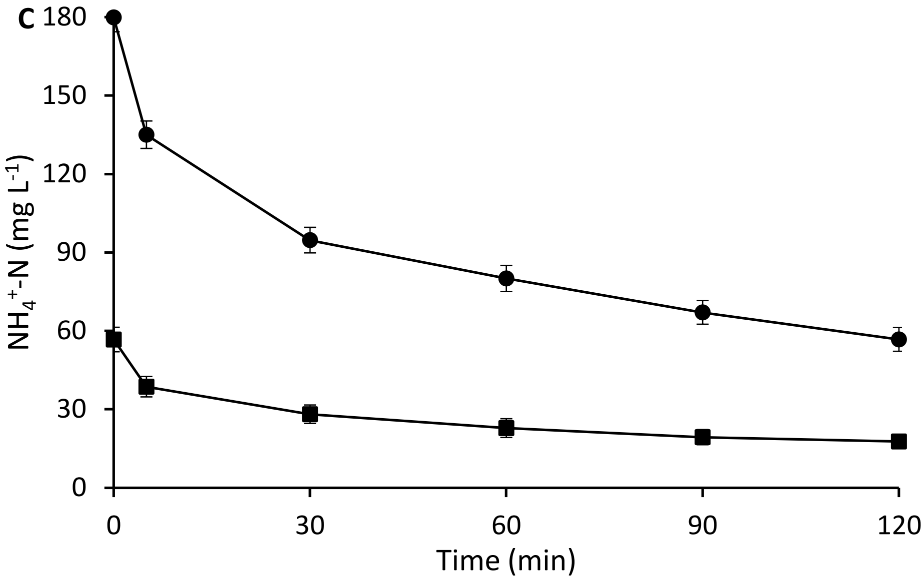
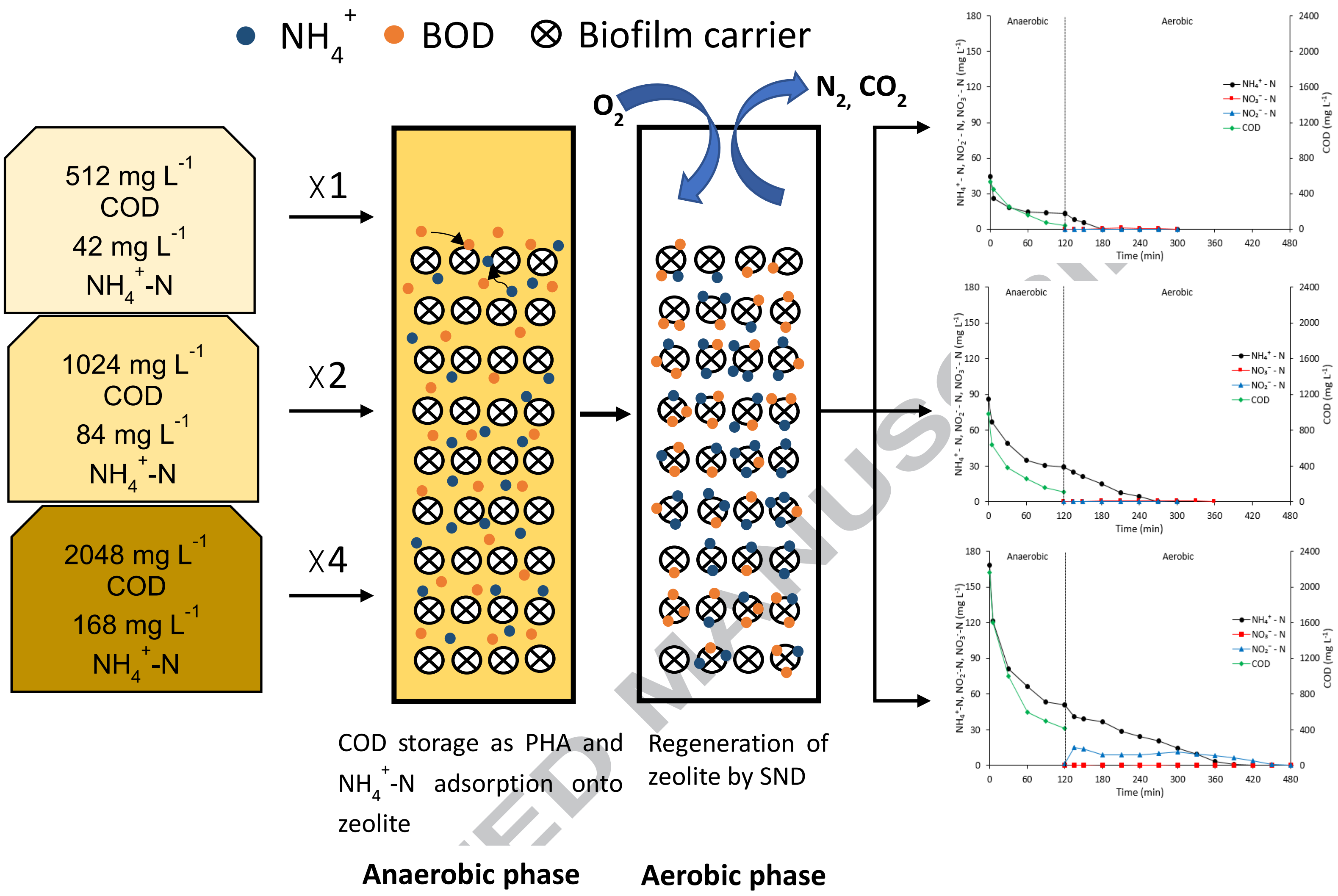


Figure 6: Treatment of different strength (A: 1x, B: 2x and C: 4x) wastewater in two successive treatment cycles: (●) treatment 1, (■) treatment 2.

Highlights

- Different strength wastewater was treated in a zeolite amended GAO biofilm system
- COD uptake rate and PHA synthesis rate increased with influent organic content
- Zeolite bioregenerated via simultaneous nitrification and PHA based denitrification
- Total treatment time did not increase proportionally to the wastewater strength
- Rapid proliferation of *Candidatus competibacter* and *Nitrospira* were observed


NH$_4^+$
BOD
Biofilm carrier
512 mg L^{-1} COD
42 mg L^{-1} NH$_4^+$-N
1024 mg L^{-1} COD
84 mg L^{-1} NH$_4^+$-N
2048 mg L^{-1} COD
168 mg L^{-1} NH$_4^+$-N
X1
X2
X4
O$_2$
N$_2$, CO$_2$
COD storage as PHA and NH$_4^+$-N adsorption onto zeolite
Regeneration of zeolite by SND
Anaerobic phase
Aerobic phase
Anaerobic
Aerobic
NH$_4^+$ - N
NO$_3^-$ - N
NO$_2^-$ - N
COD
Time (min)
COD (mg L^{-1})